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# Characterization of protective extracellular membrane-derived vesicles produced by *Streptococcus pneumoniae*

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## ABSTRACT

Extracellular vesicles are produced by many pathogenic microorganisms and have varied functions that include secretion and release of microbial factors, which contribute to virulence. Very little is known about vesicle production by Gram-positive bacteria, as well as their biogenesis and release mechanisms. In this work, we demonstrate the active production of vesicles by *Streptococcus pneumoniae* from the plasma membrane, rather than being a product from cell lysis. We biochemically characterized them by proteomics and fatty acid analysis, showing that these vesicles and the plasma membrane resemble in essential aspects, but have some differences: vesicles are more enriched in lipoproteins and short-chain fatty acids. We also demonstrate that these vesicles act as carriers of surface proteins and virulence factors. They are also highly immunoreactive against human sera and induce immune responses that protect against infection. Overall, this work provides insights into the biology of this important Gram-positive human pathogen and the role of extracellular vesicles in clinical applications.

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Biological significance
Pneumococcus is one of the leading causes of bacterial pneumonia worldwide in children and the elderly, being responsible for high morbidity and mortality rates in developing countries. The augment of pneumococcal disease in developed countries has raised major public health concern, since the difficulties to treat these infections due to increasing antibiotic resistance. Vaccination is still the best way to combat pneumococcal infections. One of the mechanisms that bacterial pathogens use to combat the defense responses of invaded hosts is the production and release of extracellular vesicles derived from the outer surface. Little is known about this phenomenon in Gram-positives. We show that pneumococcus produces membrane-derived vesicles particularly enriched in lipoproteins. We also show the utility of pneumococcal vesicles as a new type of vaccine, as they induce protection in immunized mice against infection with a virulent strain. This work will contribute to understand the role of these structures in important biological processes such as host-pathogen interactions and prevention of human disease.


## 1. Introduction

Evolutionary processes of pathogenic microbes that confer ability to survive in a host include, among other factors, the production of extracellular vesicles (EVs) derived from the outer surface, which can serve to combat host defense responses, and/or as a biophysical mechanism to release cellular stress [1]. Vesicle release is a conserved phenomenon among Gram-negative bacteria, including pathogenic and non-pathogenic species [2]. EVs have been mainly described in vitro, although numerous studies have also shown that they can act in vivo as carriers of toxins that lack canonical exporting/secretion signal sequences [3], promote cell–cell communication [4] and other biological processes. Additionally, when used as immunogens, EVs can be antigenic and induce a host immune response that protects against infection [5].

The production of EVs in Gram-positive bacteria and fungi had been overlooked until recently, largely because it was assumed that the existence of a thick, rigid cell wall would prevent the release of membrane blebs. In addition, some studies on mesosomes in streptococcal species dating to the 1960s were abandoned as they were thought afterwards to be an artifact [6,7]. During recent years, both fungi [8–11] and certain Gram-positive microorganisms [12–14] have also been found to produce EVs, suggesting that vesicle formation is a widespread process among microbes, including those with outer cell walls. Nonetheless, compared to those of Gram-negative bacteria, little is known about fungal and Gram-positive EVs, especially regarding their biogenesis and role in host-pathogen interactions. Among Gram-positive organisms, EVs have been shown to carry toxins in *Bacillus anthracis* [14] and *Staphylococcus aureus* [15,16]; fuse with host cells, inducing their death [13,15,16]; induce a proinflammatory response in vitro and in vivo in *Mycobacterium* spp. [13], and elicit a protective immune response against infection [14].

*Streptococcus pneumoniae*, also known as pneumococcus, is a Gram-positive bacterium that colonizes the human respiratory tract and has the potential to become invasive, which is associated with high morbidity and mortality rates worldwide, especially in developing countries [17]. The burden of pneumococcal disease, particularly pneumonia, is also increasing in developed countries, where it has raised major public health concerns because resistance to antibiotics has made these infections more difficult to treat [18]. Prevention by vaccination is considered as the most effective way to combat pneumococcal infections. However, the efficacy of currently licensed pneumococcal vaccines, which are based on the capsule polysaccharide, is less robust in high-risk population groups, including the elderly and those with HIV infection, particularly against pneumonia [19]. In addition, current polysaccharide-based pneumococcal vaccines do not cover all circulating serotypes, and pneumococcal conjugate vaccines have led to serotype replacement and capsular switching [20]. Moreover, they are expensive, especially for underdeveloped countries. In recent years, recombinant protein-based vaccines are being tested as potentially universal vaccines to protect against all serotypes, but such agents are not yet commercially available [21].

In this study, we demonstrate that pneumococcus produces EVs that, although resembling the plasma membrane in composition, are biologically and biochemically different. We also provide evidence that these vesicles are derived from the membrane and do not result from lysis of dead cells. Furthermore, we show that EVs are more enriched than the membrane fraction in lipoproteins. Finally, we show that EVs are highly immunogenic, and that EV immunization of mice protects against challenge with a virulent pneumococcal strain. These findings could lead to new types of vaccines to prevent pneumococcal infections.

## 2. Materials and methods

### 2.1. Mice and human sera and ethics statement

This research was performed according to the principles expressed in the Declaration of Helsinki. All human sera were obtained from pediatric patients admitted to Hospital Universitario Infantil Virgen del Rocío in Sevilla (Spain) with pneumococcal infection, corresponding to three cases of empyema in children of 32, 34 and 36 months of age, and three control sera, corresponding to healthy children of 50, 51 and 52 months of age. Written informed consent was obtained from the parents or legal guardians of the children.